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THE ACTIVITY OF STAPHYLOCOCCI IN MILK

W. H. Barnes

From the Department of Pathology and Bacteriology, University of California, Berkeley, Cal.

Staphylococci, particularly the aureus and albus types, have been isolated from a great variety of sources since they were first grown in pure culture by Rosenbach.[1] Many times several varieties have been isolated from the same source, thus causing much confusion as to their functional activities. In a study of their physiologic and biologic activities we have been considerably impressed with the various changes which they produce in milk, and consequently have made detailed studies relating to them.

Thorough investigation of bacteria includes specific studies of their morphologic, metabolic, and biologic variations, and no classification which omits one of these important factors is complete. Buchner[2] proved that fermentation processes, such as alcoholic, lactic, and acetic fermentations are processes not caused directly by bacteria themselves but by the enzymes they produce. Emil Fischer[3] claimed that there must be a similarity between the structure of enzymes and the structure which they decompose, and he compares their resemblance in structure to that necessary between a lock and a key in order that the latter may pass the lock. Pasteur[4] believed that fermentation corresponds to the respiration processes of animals, except that it is respiration without oxygen. Harden[5] believed that certain organisms bring about curdling of milk by changing the sugar content to lactic acid as $C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COOH$. He thought that different strains of organisms are distinguished by a proportion of different by-products. Savage,[6] working with B. coli, found that they converted soluble caseinogen to an insoluble casein but played no part in the initial curdling of milk. He ascribed this change to the action of an enzyme which these organisms produced. Kamm[7] has recently shown that phosphates are the essential agent for favoring gas production by bacteria, while a scant supply of minerals causes bacteria to produce lactic acid. One must therefore assume that two chemical processes are carried on as

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 \text{ and}$$
$$C_6H_{12}O_6 + H_2O \rightarrow CH_3COOH + CH_3CH_2OH + 2CO_2 + 2H_2.$$

It is conceivable that the processes mentioned above and many other chemical processes may be carried out by the various bacteria functioning in different environments. Kendall[8] defines enzymes as substances of unknown composition produced by living cells and divides them into two classes, exo-enzymes and endo-enzymes. He states that they incite specific chemical reactions and work uneconomically because they produce more utilizable

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[1] Mikroorganismen bei den Wundinfektionkrankheiten des Menschen, 1884.

[2] Chem. Ber., 1897.

[3] Holleman's Organic Chem., 1915, p. 287.

[4] Marshall's Microbiology, 1917, p. 156.

[5] Proc. of the Chem. Soc., 1903, 19, p. 48.

[6] J. Path. and Bact., 1904, 10, p. 10.

[7] Marshall's Microbiology, 1917, p. 156.

[8] Bacteriology, General Pathological, Intestinal, 1916, p. 49.

material for the organisms than the latter require. Little is known of the nature of enzymes but Mathews[9] believes that they are probably colloidal protein substances.

Coagulation of milk may be brought about by two different processes, namely, addition of excess acid and action of enzyme-rennet. A question of no little importance is whether coagulation of milk by staphylococci is effected by the production of acid or by the action of enzymes. Van Slyke and Bosworth[10] have studied the various changes that take place in milk due to acid coagulation. Normal cow's milk is slightly acid, having a P_H of about 6.2-6.4. According to a number of investigators, particularly Van Slyke and Bosworth, and Conn,[11] milk curdles when its P_H is changed to 5.4. Repeated trials using lactic acid have verified these results in this laboratory. When a sufficient amount of acid is added to milk the calcium is removed from the calcium caseinate leaving an insoluble casein in the form of a curd in the milk serum. The curd formation by the action of an enzyme in milk has been studied by Van Slyke and Publow.[12] According to these investigators, rennin first changes casein to paracasein and renders the calcium salts available for the precipitation of paracasein as calcium paracaseinate. The enzyme contained in rennet is not responsible for the coagulation of milk, but merely prepares it for chemical combinations. Curd formation is hastened by the action of excess calcium salts plus heat according to Simon.[13] Van Slyke and Publow's report shows that in the coagulation of milk the change of casein into paracasein by an enzyme consists in a hydrolytic splitting of the casein molecule, which has a molecular weight of 8888, into two equal molecules, each with a molecular weight of 4444. Heineman,[14] studying the cultural activities of hay bacilli and staphylococci has also determined that the curd is made up of an insoluble calcium paracaseinate.

My study of the enzymatic action of staphylococci in milk was conducted by attacking the problem from five different standpoints. I attempted to ascertain:

1. The amount of lactic acid necessary to form a curd.
2. The action of rennin on milk.
3. The end reaction of staphylococci on milk.
4. The daily P_H changes brought about by eight representative cultures.
5. The determination of the presence of enzymatic activity.

The experiments were repeated at three different intervals to insure constancy of results. Both desiccated and normal skim milk were tried with similar results.

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[9] Textbook of Physiological Chemistry, p. 330.

[10] "Studies Relating to the Chemistry of Milk and Casein," N. Y. Exper. Station Tech. Bull. 37, 1914; N. Y. Exp. Sta. Tech. Bull. 48, "Chemical Changes in the Souring of Milk," 1916.

[11] Tanner's Bacteriology and Mycology of Foods, p. 114.

[12] The Science and Practice of Cheese Making, 1910.

[13] Text Physiological Chemistry, 1917.

[14] "Milk," 1919.

1. *The Action of Lactic Acid in Curdling Milk.*—Tubes containing lactic acid determined which would cause a complete curd to form.

TABLE 1

AMOUNT OF LACTIC ACID CAUSING CURD TO FORM ON MILK

Quantity	Normal P_H	Amount of Lactic Acid Added	Curd	P_H
15 c c	6.4	Neutral	None	6.4
15 c c	6.4	0.1	None	6.0
15 c c	6.4	0.2	Big curd	5.8
15 c c	6.4	0.3	Partial	5.6
15 c c	6.4	0.4	Complete	5.4

The result shows the P_H at which a complete curd is formed to be 5.4. The method used was to centrifugalize the curd, throw it to the bottom of the tube and then test the supernatant whey.

2. *Action of Rennet on Milk.*—Milk treated in the following manner with liquid rennet made by Jno. Wyeth and Bro. To tubes of milk containing 15 c c, varying amounts of rennet were added and the mixture kept at 37.5 C. Three-tenths c c of the liquid rennet were found to be the least that would give a solid curd in 24 hours. Curd formation could be produced immediately by heating over a flame. In either case there was no change in the P_H.

3. *The End Result of Growing Staphylococci in Milk.*—One hundred and eighty cultures of staphylococci, consisting of 8 St. citreus and the remainder about equally divided between the aureus and albus types, were used in this work. No attempt was made to subdivide either of the aureus or albus types on a basis of variation in the quantity of pigment production, although a difference of intensity of pigment was observed, particularly in the St. aureus group. These cultures were isolated from a wide variety of pathogenic sources, both human and veterinary. They were supplied by Cutter's Biologic Laboratories, the Mulford Laboratories, and from various hospitals about the San Francisco bay region. Repeated cultivation in milk, with brom-cresol-purple used as an indicator, gave 5 different variations. The following varieties were observed:

1. No curd formation with no P_H change.
2. Curd formation with a slight decrease in P_H.
3. No curd formation with a slight P_H decrease.
4. Curd formation with a maximum P_H decrease.
5. Curd formation plus curd digestion with increase in P_H.

The curds of the second and fourth cases when first formed appeared to occupy all the space primarily occupied by the milk, but subsequently broke away from the walls of the tube as if they were liquefying. It was found, however, that by centrifugalizing them there was no apparent change in actual quantity but that the colloidal mass of precipitated casein simply contracted down and squeezed out the whey. The majority of both aureus and albus cultures, as will be observed, belong to the third and fourth types. Table 2 shows the results of 152 cultures of staphylococci when grown in milk.

TABLE 2

RESULTS OF GROWING STAPHYLOCOCCI IN MILK

Action on Milk	Staph. aureus	Staph. albus	Staph. citreus
1. No change in milk	4	2	3
2. Curd formation plus slight P_H decrease	8	16	0
3. No curd formation plus slight P_H decrease	7	15	1
4. Curd formation plus minimum P_H decrease	45	46	3
5. Curd formation and digestion plus P_H increase	8	2	0

4. *Tests with Eight Representative Cultures.*—The results given above indicate that various metabolic functions are performed by different types of staphylococci. Daily P_H readings, continuing through a period of 4 weeks, were carried out in an attempt to interpret the functional changes of these organisms in a special environment. A sufficient number of tubes of sterilized milk, each containing 15 c c, was inoculated with 0.1 c c of a 24-hour broth culture so that a single tube could be examined and discarded each day to avoid liability of contaminations. Tests were made with normal fresh skimmed milk and with desiccated skimmed milk. The desiccated skimmed milk gave equally good results and was simpler to handle. During a period of 12 weeks, 3 different series of tests were carried out. Since there were no demonstrable changes after the 7th day, the first to the 7th day (Sunday excluded), 14th, and 21st days only are reported. Table 3 gives the combined results.

If we compare the results in table 1 with those in table 3, we will observe that coagulation began with the same P_H as that of lactic acid coagulation, but instead of partial coagulation as given by the lactic acid we have a complete coagulation given by the activity of the organisms in milk. We were therefore compelled to recognize some other agency causing milk coagulation besides that of acid production by staphylcocci. Naturally we turned our attention to enzymes and endeavored to find out under what conditions staphylococci produce enzymes capable of milk coagulation.

TABLE 3

PH OF MILK BEFORE INOCULATION 6.2 TO BROM-CRESOL-PURPLE

Number and Type of Culture	PH on Number of Days Indicated								
	1	2	3	4	5	Sunday 6	7	14	21
50 St. albus........	6.1	6.0	6.1	6.2	6.2	..	6.1	6.1	6.1
120 St. albus........	6.1	6.1	6.1	6.1	6.1	..	6.1	6.1	6.1
122 St. albus........	6.0	6.0	5.9	5.9	5.9	..	5.9	5.9	5.9
106 St. aureus.......	5.9	5.8	5.8*	5.7*	5.7*	..	5.7*	5.7*	5.7*
14 St. albus........	5.9*	5.8*	5.7*	5.6*	5.5*	..	5.4*	5.4*	5.4*
109 St. albus........	5.7*	5.7*	5.7*	5.6*	5.6*	..	5.5*	5.7*	5.7*
61 St. albus........	6.0*	5.8*	5.8*	5.7*	5.7*	..	5.6*	5.6*	5.6*
118 St. albus........	6.1	6.1	6.1	6.2	6.4	..	6.4	6.4	6.3

* Curd formation complete for the whole tube.

5. *Determination of Enzymatic Activity.*—Three rapidly coagulating strains were chosen for this work. Three cultures of each were grown in bouillon and also in milk for 96 hours. One bouillon culture was killed by formalin. The remaining bouillon cultures were centrifugalized and both supernatant fluid and sediment were saved and 1% formalin added. Sterility tests were made after 3 days to insure the death of all organisms. The sediment was resuspended in normal salt solution equal to the amount of bouillon poured off. The milk cultures that were clotted were likewise centrifugalized, the whey preserved, the centrifugalized curd resuspended in bouillon, and incubated for 3 days longer. These culture were then centrifugalized and the supernatant bouillon from the milk curd was saved. Both the whey and the milk curd bouillon were sterilized by 1% formalin, as in the preceding bouillon cultures. Sterility tests were made on agar slants the same as for the bouillon cultures. When the sterility of the above broth and milk solutions and suspensions was established, 1 c c, 2 c c, and 3 c c each were added to tubes of milk containing 10 c c and incubated at 37.5 C for 6 days. Daily observations were made. As will be seen, this gave 3 tests for each solution or suspension. Table 4 shows the type of solution and its enzymatic activity on milk.

Table 4 shows that milk was clotted by the sterilized supernatant whey from cultures 14 and 34, and by supernatant bouillon from bouillon-milk curd culture from culture 14. These clots were subcultured on agar slants and incubated for 48 hours but gave no growth. It thus seems reasonable to conclude that the clotting of milk in these cases must be due to the enzyme produced by the staphylococci. No attempt was made to establish any relationship between a proteolytic and a rennet-like enzyme. Other experiments in progress show cultures 14 and 34 to be rapid liquefiers of gelatin and 106 to be a slow liquefier. The results further demonstrate the possibility of the production of an enzyme by staphylococci.

TABLE 4

ENZYMATIC ACTIVITY OF VARIOUS SOLUTIONS ON MILK

	Quantity of Culture 14			Quantity of Culture 34			Quantity of Culture 106		
	1 c c	2 c c	3 c c	1 c c	2 c c	3 c c	1 c c	2 c c	3 c c
Killed broth culture	—	—	—	—	—	—	—	—	—
Supernatant centrifugalized broth	—	—	—	—	—	—	—	—	—
Saline suspension of sediment from broth culture	—	—	—	—	—	—	—	—	—
Supernatant whey from milk culture	—	+	+	—	+	—	—	—	—
Supernatant broth from milk curd culture	—	—	+	—	—	—	—	—	—

—, no clot formed; +, solid clot formed.

DISCUSSION AND CONCLUSIONS

The results of this investigation demonstrate that milk is a valuable factor in the classification of staphylococci on account of the various reactions obtained. The phenomenon that bears the most interest in the problem is the question of metabolic activity, particularly among the cultures that caused milk coagulation. Is coagulation due to acid production, direct action of the organisms on the milk, to the production of an enzyme or to some combination of three factors? Judging from the P_H titer of the curdled tube of milk, one might consider it to be a group of factors, such as acid plus direct action, acid plus enzyme action or enzyme action alone. Evidence thus far at hand shows that when acid and direct action of the organisms are eliminated, the specific means are used, as for example, treating the milk with whey from killed coagulated milk cultures, a curd may be produced in sterile milk, thus proving the production of a specific milk curdling enzyme. While further work is in progress on this problem, we feel justified in making the claim that certain strains of staphylococci not related by pigment production are capable of producing a milk curdling enzyme.